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SHORT COMMUNICATION

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Ethylene oxide-protein adduct formation in humans: influence of glutathione-*S*-transferase polymorphisms

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Abstract Objective: The influence of the polymorphic human glutathione-*S*-transferase (GST) T1 and M1 genotypes (classified as "conjugators" and "nonconjugators") on the biological effects of nonoccupational ethylene oxide exposure as reflected by the formation of globin N^2-hydroxyethylvaline adducts was investigated. Specific attention was paid to smoking as a potential source of exposure. A total of 27 Caucasian subjects, including 10 women and 17 men, participated in the study. Volunteers were grouped as smokers, i.e., 6 subjects (5 male, 1 female), and nonsmokers, i.e., 21 subjects (12 male, 9 female). The regular cigarette consumption in the smoker group ranged from 10 to 25 cigarettes/day. **Methods:** The amount of N^2-hydroxyethylvaline (HEV) bound to the *N*-terminal valine in human globin was determined following a procedure described by Bader and co-workers and the Deutsche Forschungsgemeinschaft. The GST genotypes were determined by a polymerase chain reaction (PCR) analysis outlined by Bell and colleagues (with β-globin serving as an internal standard) and Kempkes and co-workers (coamplification of the GSTT1 fragment). **Results:** The median level of HEV detected in the smoker group was 280 pmol/g globin as compared with the median value of 50 pmol/g globin recorded for the nonsmokers, indicating that ethylene oxide intake from cigarettes may result in a ~5-fold higher overall HEV level in smokers in comparison with nonsmoking individuals. No dose-effect correlation was observed between daily cigarette consumption and the resulting HEV levels. Moreover, the individual GSTT1 or GSTM1 genotype did not influence the HEV level in smokers. The subgroup of nonsmoking GSTT1 conjugators revealed a median HEV value of 46 pmol/g globin as compared with the median value of 92 pmol/g globin found in the nonconjugator subgroup. Subjects with the GSTM1 gene had a median HEV value of 55 pmol/g globin, whereas subjects with the gene deletion had a median HEV value of 44 pmol/g globin. The 2-fold increase in the median HEV value detected in GSTT1 non-conjugators as compared with GSTT1 conjugators indicates an influence of GSTT1 on the globin HEV levels. This hypothesis was found to be significant in the nonparametric Mann-Whitney *U*-test for independent samples ($P < 0.002$, two-sided). The same test was applied to evaluate the influence of GSTM1 on the globin HEV levels. No significant influence was observed ($P > 0.10$; two-sided). **Conclusions:** This result is in accordance with the finding that ethylene oxide is a substrate for GSTT1 but not for GSTM1. In addition, this study demonstrates a clear influence of genetically determined GSTT1 status on biological effects, e.g., protein adduct formation after non-occupational ethylene oxide exposure. In smokers, however, a modulating influence of GSTT1 status was not observed.

Key words Polymorphism · Glutathione-*S*-transferase T1 · Glutathione-*S*-transferase M1 · N^2-Hydroxyethylvaline · Protein adducts

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Introduction

Ethylene oxide is a widely used industrial chemical. Although occupational exposure is regulated and monitored (Angerer et al. 1998), environmental exposure due to different sources, such as biogenic production of ethylene oxide from ethene in plants and bacteria (Hou et al. 1983) and involuntary release of the chemical from industrial processes and products (BUA 1993), should not be neglected. Ethylene oxide is easily absorbed upon

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inhalation, ingestion, and skin contact. It is detoxified mainly by hydrolysis to ethanediol and by glutathione conjugation as the minor metabolic pathway (Jones and Wells 1981; Martis et al. 1982).

Föst and co-workers (1991) demonstrated the conjugation of ethylene oxide by a human glutathione-*S*-transferase in blood. This activity was later attributed to glutathione-*S*-transferase T1 (GSTT1) from erythrocytes (Schröder et al. 1992, 1996). The enzyme activity displays polymorphism; approximately three-quarters of the Caucasian population are "conjugators," whereas one-quarter, the "nonconjugators," lack this activity (Hallier et al. 1993). Lack of enzyme activity is due to a gene deletion, which can be detected by polymerase chain reaction (PCR) analysis of lymphocyte DNA (Pemble et al. 1994). The polymorphism should be considered in risk assessment (Bolt 1996), as part of the population may have a higher susceptibility to toxic effects of compounds such as ethylene oxide due to the genetically determined lack of metabolic activity.

The aim of our study was to investigate in a Caucasian population without occupational exposure whether there was an influence of the GSTT1 genotype on the biological effects of ethylene oxide as reflected by the formation of globin N^2-hydroxyethylvaline adducts (Bader et al. 1995). Parallel investigations were performed, focusing on another polymorphic human glutathione-*S*-transferase (GSTM1), and specific attention was paid to smoking as a potential source of exposure.

Subjects and methods

Subjects

A total of 27 Caucasian subjects, including 10 women and 17 men, participated in the study. None of the subjects had had occupational contact with ethylene or ethylene oxide, which could result in elevated levels of N^2-hydroxyethylvaline. Smoking habits, known to influence the N^2-hydroxyethylvaline levels in erythrocytes (Bader et al. 1995), were evaluated by an interview. Volunteers were grouped as smokers (6 subjects: 5 male, 1 female) and non-smokers (21 subjects: 12 male, 9 female). The regular cigarette consumption in the smoker group ranged from 10 to 25 cigarettes/day.

Chemicals

All chemicals used were of the highest analytical grade available and were purchased from Merck and Fluka, Germany, except for formamide (Amersham Life Science, Germany), DL-pipecolic acid (Sigma, Germany), and N^2-hydroxyethylvaline-leucine-anilide (Bachem Biochemica, Germany).

Blood sampling and isolation of globin

Whole blood (5 ml) was collected from each subject by venipuncture using syringes containing ethylenediaminetetraacetic acid (EDTA; Sarstedt, Germany). The blood was centrifuged for 10 min at 800 *g* and the supernatant plasma was discarded. The erythrocytes were washed three times with 5 ml of 0.9% NaCl solution. Hemolysis was achieved by the addition of 5 ml of distilled water and freezing of the samples below −18 °C.

For the isolation of approximately 150 mg of globin from a sample, 2 ml of hemolysate was added to 12 ml of a 50-mM hydrochloric acid solution in 2-propanol. The samples were stored for at least 1 h at 4 °C and were subsequently centrifuged for 10 min at 3500 *g*. Ethyl acetate (8 ml) was added slowly to the supernatant and the solution was stored overnight at 4 °C. The precipitated globin was spun down for 10 min at 3500 *g* and washed three times with 5 ml of ethyl acetate. Finally, the globin was washed with 5 ml of *n*-hexane and dried in a vacuum desiccator.

Blood sampling and leukocyte DNA isolation

Whole blood (10 ml) was collected from each person by venipuncture using EDTA-containing syringes (Sarstedt, Germany). The blood was centrifuged for 10 min at 400 *g* and the supernatant plasma was discarded. Erythrocytes were hemolyzed and the leukocytes recovered by centrifugation (400 *g*, 10 min). The DNA was extracted according to a procedure outlined by Miller et al. (1988).

Determination of N^2-hydroxyethylvaline

The amount of N^2-hydroxyethylvaline (HEV) bound to the *N*-terminal valine in human globin was determined essentially following a procedure described by Bader et al. (1995) and the Deutsche Forschungsgemeinschaft (DFG 1997). In a modified Edman degradation procedure the alkylated valine was allowed to react with pentafluorophenyl isothiocyanate to yield the pentafluorophenyl thiohydantoin derivative of HEV, which was detected and quantified by gas chromatography/mass spectrometry (GC/MS) analysis. The reliability of the method was checked by determination of the precision in the series: 7.5% for 8 samples with a mean concentration of 95 pmol HEV/g globin. The precision from day to day was 10.7% for 10 samples with a mean HEV concentration of 152 pmol/g globin. The detection limit was 0.1 μg/l (19 pmol/g).

GC/MS conditions

The following conditions were used for GC: gas chromatograph – Hewlett Packard 5890 series II; capillary column – J&W DB-17HT, 30 m × 0.25 mm, 0.15-μm film thickness; carrier gas – He 5.0; column head pressure – 15 psi; and temperatures – injector 280 °C, column 100 °C, 7 °C/min to 240 °C, 20 °C/min to 280 °C (11 min). The following conditions were applied for MS: mass spectrometer – Hewlett Packard 5989 A; temperatures – transfer line 300 °C, quadrupole 100 °C, ion source 200 °C; ion source pressure – 2×10^{-5} Torr; ionization mode – electron impact (EI, 70 eV); and detection mode – selected ion monitoring (SIM), dwell time 100 ms/ion, electron multiplier 2600 V.

Analytical determination

In all, 1 μl of each sample was injected splitless into the GC. The pentafluorophenyl thiohydantoin derivatives of HEV and the internal standard pipecolic acid (PCA) were identified by their retention times of 17.7 min for PCA and 18.2 min for HEV and by two characteristic masses – 308/350 amu for HEV and 336/308 amu for PCA. Quantification was based on the ratio of the peak areas obtained from the ions at *m/z* 308 (HEV) and 336 (PCA), respectively. The amount of HEV was calculated from the calibration curve.

Genotyping of GSTT1 and GSTM1

For the PCR analysis of the leukocyte DNA samples, primers of the GSTT1 and GSTM1 genes were used as originally described by Pemble et al. (1994) and Bell et al. (1992). The PCR method was carried out according to Bell et al. (1992; with β-globin serving as

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an internal standard) and Kempkes et al. (1996; coamplification of the GSTT1 fragment). PCR products were separated on a 2% agarose gel and made visible by ethidium bromide staining.

Results and discussion

Individual blood samples were collected and analyzed for globin HEV levels as described above. DNA was extracted from the leukocytes and analyzed by PCR for GSTT1 and GSTM1 genes (for details see subjects and methods). The results are presented in Tables 1 and 2.

The median value recorded for globin HEV in the smoker group was 280 pmol/g globin as compared with the median value of 50 pmol/g globin noted for the nonsmokers. No dose-effect correlation was observed between daily cigarette consumption and the resulting HEV levels.

The distribution of subjects with the GSTT1 genotype, called "conjugators," and subjects without the GSTT1 gene, called "nonconjugators," was 76% versus 24% in the nonsmoker group. This reflects the expected 3:1 distribution among the Caucasian population and is in excellent agreement with previous data (Hallier et al. 1993). In the smoker group the percentage of GSTT1 nonconjugators (33%) versus conjugators (67%) was slightly elevated, most likely due to the small number of participating smoking subjects.

In the nonsmoker group the GSTM1 genotype was detected in 43% of subjects versus the 57% who showed a deletion of the entire GSTM1 gene. The presence of the GSTM1 gene was demonstrated in the DNA of 50% of the smokers, whereas it was absent in the remaining 50% of the individual genomes. This observation is in good agreement with the findings of Bell et al. (1992), who demonstrated 51% null-allele bearers in a North Carolina population.

Thus, the distribution of the GSTT1 and GSTM1 genotypes in both the smoker and nonsmoker groups was similar to that previously observed in Caucasian populations. This provided a basis for investigation of the influence of specific GST genotypes on the individual globin HEV levels.

In smokers the median globin HEV value recorded for the GSTT1 nonconjugator subgroup was 272 pmol/g globin as compared with the value of 280 pmol/g globin noted for the conjugator subgroup. The GSTM1 conjugator subgroup had a median globin HEV value of 292 pmol/g globin versus the median HEV value of 268 pmol/g globin detected in the nonconjugator subgroup. Although daily cigarette consumption varied and the number of smoking participants in the study was small, the data suggest that ethylene oxide intake from cigarettes may result in a ~5-fold higher overall HEV level in smokers in comparison with nonsmoking individuals. Moreover, the individual GSTT1 or GSTM1 genotype apparently does not influence the HEV level.

The subgroup of nonsmoking GSTT1 conjugators revealed a median HEV value of 46 pmol/g globin as compared with the median HEV value of 92 pmol/g globin found in the nonconjugator subgroup. Subjects with the GSTM1 gene demonstrated a median HEV value of 55 pmol/g globin, whereas subjects with the gene deletion had a median HEV value of 44 pmol/g globin.

The 2-fold increase in the median HEV value detected in GSTT1 nonconjugators as compared with GSTT1 conjugators suggested an influence of the GSTT1 on the globin HEV levels. This hypothesis was tested for significance using the nonparametric Mann-Whitney *U*-test for independent samples (H_1: HEV levels of GSTT1 nonconjugators > HEV levels of GST conjugators; H_0: HEV levels of GSTT1 nonconjugators = HEV levels of GSTT1 conjugators). The HEV levels detected in the GSTT1 nonconjugator group were significantly higher than those found in the conjugator group ($P < 0.002$, two-sided). The same test was applied to evaluate the influence of GSTM1 on the globin HEV levels. No significant difference was observed between the HEV levels recorded for the GSTM1 non-conjugator and conjugator subgroups ($P > 0.10$; two-sided).

Table 1 Parameters determined in the group of nonsmokers

Individual	Sex	Genotype GSTT1	HEV (pmol/g globin)	Genotype GSTM1
1	M	−	176	+
2	M	−	170	+
3	F	−	92	+
4	F	−	72	+
5	F	−	70	−
6	M	+	61	−
7	F	+	58	+
8	F	+	57	+
9	F	+	53	−
10	M	+	52	+
11	M	+	50	+
12	M	+	46	−
13	F	+	46	+
14	M	+	45	+
15	F	+	44	−
16	M	+	43	+
17	M	+	39	−
18	M	+	37	−
19	M	+	35	−
20	M	+	30	+
21	F	+	22	−

Table 2 Parameters determined in the group of smokers

Individual	Sex	Cigarettes/day	Genotype GSTT1	HEV (pmol/g globin)	Genotype GSTM1
1	M	15	+	471	−
2	M	25	−	309	+
3	M	25	+	292	+
4	F	10	+	268	−
5	M	10	+	264	−
6	M	10	−	234	+

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This result is in accordance with data reviewed by Bolt (1996), confirming ethylene oxide as a GSTT1 substrate, whereas no affinity was seen for GSTM1. Moreover, a clinical study by Grammer et al. (1984) on hemodialysis patients revealed interindividual differences: subjects afflicted with immediate-type allergic reactions during treatment that included the use of ethylene-oxide-sterilized material ("reactors") had significantly higher levels of serum albumin-ethylene oxide adducts than did those without allergic reactions ("non-reactors"). Since major differences in the sterilization of the materials and the resulting exposure to ethylene oxide is unlikely, it may be speculated that the interindividual differences in allergic reactions could have been due to the enzyme polymorphism. Fuchs and co-workers (1994) investigated DNA damage in subjects occupationally exposed to ethylene oxide using the "alkaline elution" technique. Two subgroups with differential susceptibility to ethylene oxide were detected in the nonsmoker group of study participants. Although both Grammer et al. (1994) and Fuchs et al. (1994) had indications for subgroups on the basis of molecular dosimetry, the existence of these subgroups could not be linked to different genetically determined mechanisms of detoxification. Our data clearly suggest such a link between globin HEV levels and polymorphic GSTT1. This view is further supported by previous results of biological effect monitoring, demonstrating in vitro a correlation between the induction of sister chromatid exchanges in lymphocytes and the GSTT1 conjugator status after ethylene oxide exposure (Hallier et al. 1993).

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